수의학석사학위논문

개 지방유래 중간엽 줄기세포를 이용하여 만든 동결보존 젤라틴 골분화 세포시트의 골 형성능

In Vitro Osteogenic Ability of Cryopreserved Gelatin-induced Osteogenic Cell Sheets Using Canine Adipose Tissue-derived Mesenchymal Stromal Cells

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ABSTRACT

Gelatin-induced osteogenic cell sheets (GCSs) have been shown to possess osteogenic transdifferentiation capabilities and high cell sheet quality. However, the clinical applications of GCSs are limited owing to the lengthy cell preparation period.

Cryopreservation of GCSs may allow clinicians to use cell sheets whenever they need. In this study, we evaluated the effects of the freeze-thaw process on GCSs. Fresh GCSs showed 3–4 layers with abundant ECM formation; however, GCSs after freeze-thawing decreased by 1–2 layers. Cryopreserved GCSs right after thawing showed no significant differences in cell viability compared with fresh GCSs. However, cryopreserved GCSs did not proliferate in culture after freeze-thawing. The mRNA expression levels of runt-related transcription factor 2 and β-catenin did not differ between fresh and cryopreserved GCSs on day 0, but showed significantly lower expression on day 2 ($p < 0.05$). However, the mRNA expression of osteopontin increased significantly on day 2 after freeze-thawing compared with that of fresh GCSs. The level of bone morphogenic protein-7 did not differ between groups. Mineralization was confirmed by further culturing after freeze-thawing. This data suggested that cryopreserved GCSs had osteogenic potential and the ability to maintain sheet morphology. This technique could be available for clinical applications.

Key words: gelatin-induced osteogenic cell sheets, cryopreservation, osteogenic potential.

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CONTENTS

I. INTRODUCTION

Mesenchymal stromal cells (MSCs) are multipotent cells used for regenerative medicine due to their ability to transdifferentiate (Parekkadan *et al.*, 2010; Uccelli *et al.*, 2008). Cryopreservation of MSCs has been studied to shorten the preparation time and improve the efficiency of clinical use (Bruder *et al.*, 1997; Spurr *et al.*, 2002). Systemically injected MSCs migrate to the target site through the homing effect (Devine *et al.*, 2003). However, some studies have reported that cryopreservation of MSCs decreases the viability, differentiation ability, and binding of the cells (Pal *et al.*, 2008; Chinnadurai *et al.*, 2014; Francois *et al.*, 2012). Additionally, freeze-thawing can decrease the homing effect, biodistribution properties, and fibronectin connection of MSCs after infusion (Chinnadurai *et al.*, 2008).

Whereas, osteogenic-differentiated MSC sheets (OCSs) have been shown to have osteogenic potential in vitro (Akahane *et al.*, 2008; Guo *et al.*, 2015; Ma *et al.*, 2010; Wei *et al.*, 2012). Additionally, application of OCSs promotes bone repair (Uchiyama *et al.*, 2011; Pirraco *et al.*, 2011; Inagaki *et al.*, 2013). Freeze-thawed OCSs have osteogenic potential and produce a mineralized matrix at bone defect sites (Kura *et al.*, 2016).

Unlike systemically injected MSCs, cell sheets can be maintained in the localized region and function sufficiently without a sharp decrease in efficacy (Kelm *et al.*, 2010).

Gelatin-induced osteogenic cell sheets (GCSs) have been reported to have good osteogenic transdifferentiation capabilities and cell proliferation rates and form cell sheets more easily than OCSs (Kim *et al.*, 2017). Cryopreservation of GCSs can shorten the cell preparation time, allowing the sheets to be used immediately.

Accordingly, in the present study, I evaluated the effects of freeze-thawing of GCS on viability and osteogenic ability of canine adipose tissue-derived MSCs (Ad-MSCs).

II. MATERIALS AND METHODS

1. Isolation and cultivation of canine Ad-MSCs

Canine Ad-MSCs were isolated as previously reported (Ryu *et al.,* 2009). Adipose tissue from subcutaneous fat of the gluteal region of 2-year-old beagle dogs was collected aseptically. All procedures for animal experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-150423-6), Korea. Harvested tissues were washed with Dulbecco's phosphate-buffered saline (DPBS; Gibco, Grand Island, NY, USA) and immersed in 1 mg/mL collagenase type I (Sigma-Aldrich, USA) at 37°C for 2 h. After treatment, the samples were washed with DPBS followed by centrifuging at 4°C and 980 × *g* for 10 min. The pellets of the stromal vascular fraction (SVF) were resuspended, filtered through 100 μm nylon mesh, and incubated overnight in low-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone, USA) with 10% fetal bovine serum (FBS; Gibco BRL, USA) at 37°C in a humidified atmosphere containing 5% CO_2. After 24 h, the samples were washed with PBS to remove residual red blood cells and unattached cells. The medium was changed every 2 days, and the cells were subcultured after reaching 90% confluence.

2. Preparation of GCSs

GCSs were prepared as previously described (Kim *et al.,* 2017). Briefly, Ad-MSCs (5×10^5 cells) at passage 3 were seeded on 100-mm dishes, 6-well plates, or 12-well plates according to the experiment. Cells were cultured in low-glucose DMEM with 10% FBS and 1% penicillin/streptomycin (PS; HyClone). After reaching 70–80% confluence, the basal medium was replaced with high-glucose DMEM with 10% FBS, 1% PS, 15 μg/ml L-ascorbic acid 2-phosphate (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich), 0.1 μM dexamethasone (Sigma- Aldrich), and 0.02 g/mL gelatin powder (Sigma-Aldrich). The cell medium was changed every 2 days, and cells were harvested at 10 days after differentiation.

3. Preparation of cryopreserved GCSs

Cryopreserved GCSs were prepared using the slow-freezing method (Kura *et al.,* 2016). After 10 days of differentiation, cell sheets were harvested using a cell scraper and moved to 2 mL cryovials (cryogenic vial; BD Falcon). Each cryovial contained 500 μL FBS, 500 μL gelatin-containing basal medium, and 100 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich). Cryovials containing GCSs were frozen in a freezing container. The temperature of the container was slowly decreased at 1°C/min from 4°C to -80°C using a cryo-freezing container (NALGENE Cryo 1°C Freezing Container; Sigma-Aldrich). After 24 h, the samples were moved to a liquid nitrogen tank. After freezing for 1 week, cells were fully thawed at 37°C in a water bath. The thawed GCSs were then cleaned twice in PBS because use in further experiments.

4. Cell viability assay

The viability and proliferation after thawing was determined using a previously reported method based on tetrazolium reductase activity (Cell Counting Kit-8 [WST-8]; Dojindo; Kumamoto, Japan) (Shimizu *et al.,* 2013; Kito *et al.,* 2005). First, 100 μL of WST-8 solution was added to 1 mL culture medium, the samples were incubated for 2 h, and the absorbance was measured using a spectrophotometer at 450 nm. A linear relationship (correlation $R^2 = 0.9981$) was confirmed between the absorbance measured and the number of diluted cells. The GCSs made in 12-well plates were cryopreserved. The absorbance was measured immediately after thawing and then on days 1 and 2 after thawing to evaluate the proliferation of cells when recultured in gelatin-containing basal medium.

5. Histologic examination of GCSs

GCSs were easily detached from the plates, and the sheets were fixed with 4% paraformaldehyde and embedded in paraffin. Sections (5 μm thick) were prepared, rehydrated, and stained with hematoxylin and eosin (H&E; Sigma-Aldrich).

6. RNA isolation and real-time quantitative polymerase chain reaction (PCR)

RNA was isolated using a Hybrid-R RNA Extraction Kit (GeneAll, Seoul, Korea). Synthesis of complementary DNA was performed using a PrimeScript II First-strand cDNA Synthesis Kit (Takara, Otsu, Japan). Real-time PCR was then performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Green Mix (Enzo Life Science, Farmingdale, NY, USA) was used to detect gene expression. The expression levels of mRNAs were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and quantified using the ΔΔCt method (Livak *et al.,* 2001). All data were compared with MSCs, which were used as the control and set at 1.0. The primer sequences of the target genes, including runt-related transcription factor 2 (Runx2), β-catenin, osteopontin (OPN), and bone morphogenetic protein 7 (BMP-7), are shown in Table 1.

Table 1. Primers for real-time quantitative polymerase chain reaction.

Gene	Primer sequence (5'-3')	
	Forward	**Reverse**
Runx2	TGTCATGGCGGGTAACGAT	TCCGGCCCACAAATCTCA
β-catenin	TACTGAGCCTGCCATCTGTG	ACGCAGAGGTGCATGATTTG
OPN	GATGATGGAGACGATGTGGATA	TGGAATGTCAGTGGGAAAATC
BMP-7	TCGTGGAGCATGACAAAGAG	GCTCCCGAATGTAGTCCTTG
GAPDH	CATTGCCCTCAATGACCACT	TCCTTGGAGGCCATGTAGAC

Runx2, runt-related transcription factor 2; OPN, osteopontin; BMP-7, bone morphogenetic protein-7; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

7. Detection of mineralization

Cells cultured in 6-well plates were used for this assay. Unfrozen fresh GCSs and slow-freezing GCSs were compared to evaluate the amount of calcification. All cell sheets were harvested in the same manner and washed with DPBS twice before measurement. After washing, cell sheets were fixed in 4% paraformaldehyde (Wako) for 10 min at 37°C. The samples were then washed with distilled water, treated with 2% Alizarin red staining (ARS; pH 4.2), and then incubated for 20 min with shaking. After aspirating the dye, the wells were washed thoroughly with distilled water, and 1 mL of 10 mM (10%) cetylpyridinium chloride was added. Plates were shaken for 80 min, 100 μL of the solution was added to each well, and the absorbance was measured at 570 nm (Gregory *et al.*, 2004).

8. Statistical analysis

Data are presented as means ± standard deviations (SDs). All measurements were performed using SPSS version 23.0 (SPSS Inc., Chicago, IL, USA). The significance of differences between groups was analyzed using Kruskal-Wallis tests and Mann-Whitney U tests. Differences with p values of less than 0.05 were considered significant.

III. RESULTS

1. Viability of cryopreserved GCSs

The numbers of viable cells in fresh and cryopreserved GCSs were 148,125 ± 20,881 (n = 16) and 113,672 ± 34,500 (n = 15), respectively. There were no significant differences between fresh and cryopreserved GCSs (Fig. 1).

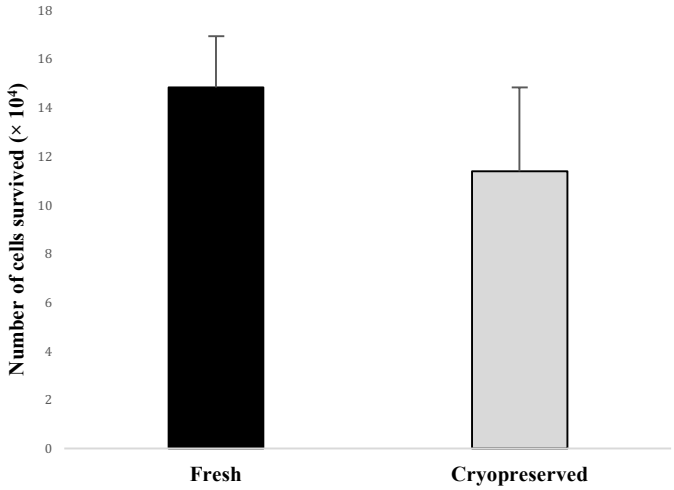

Figure 1. Comparison of numbers of viable cells between fresh and cryopreserved GCSs immediately after thawing.

2. Proliferation rates of cryopreserved GCSs after culture

The number of viable cells in cryopreserved GCSs decreased during further cultivation; however, there were no significant differences between days of culture (Fig. 2).

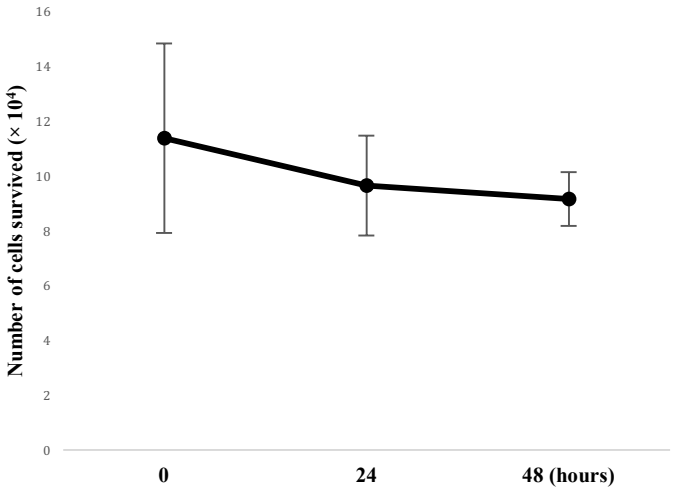

Figure 2. Changes in viable GCSs after thawing.

3. Comparison of thickness between fresh and cryopreserved GCSs

Fresh GCSs showed 3–4 layers with abundant ECM formation in H&E staining. The GCSs after freeze-thawing maintained shape but exhibited only 1–2 layers (Fig. 3).

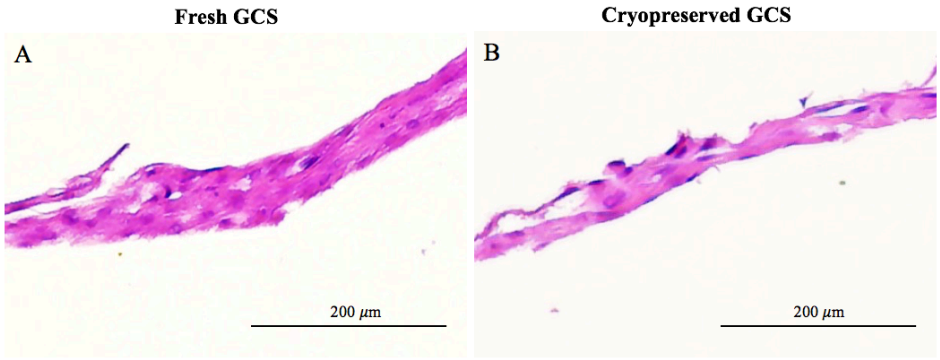

Figure 3. Cross-sections of GCSs after H&E staining. Scale bar: 200 μm. (A) Fresh GCSs, (B) cryopreserved GCSs. Both were harvested after 10 days of culture.

4. Expression of osteogenic markers of cryopreserved GCSs

The mRNA expression levels of Runx2 and β-catenin were not different between fresh and cryopreserved GCSs on day 0, but showed significantly lower expression on day 2 ($p < 0.05$; Fig. 4A, B). The mRNA expression of OPN increased significantly on day 2 after freeze-thawing as compared with that in fresh GCSs ($p < 0.05$; Fig. 4C); however, the level of BMP-7 did not differ (Fig. 4D).

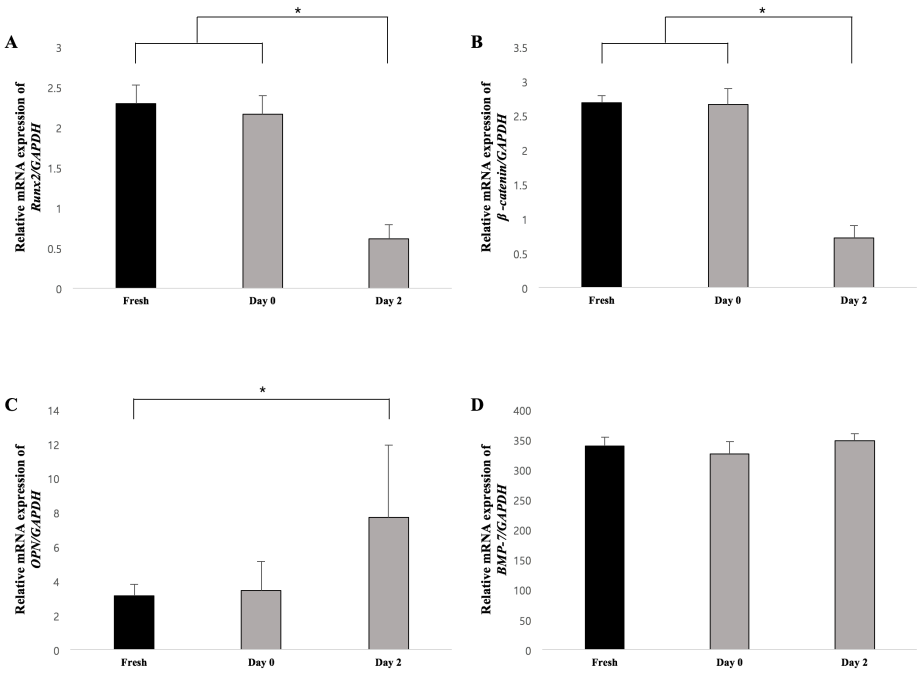

Figure 4. Osteogenic mRNA expression in fresh (black) and cryopreserved (gray) GCSs. Cryopreserved GCSs were examined on days 0 and 2 of cultivation. mRNA expression levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (A) Runt-related transcription factor 2 (Runx2), (B) β-catenin, (C) osteopontin (OPN), and (D) bone morphogenetic protein-7 (BMP-7). * $p < 0.05$ compared with the fresh group.

5. ARS staining for mineralization

The absorbance of ARS staining for mineralization was significantly increased on days 5 and 11 compared with that on day 0 ($p < 0.05$, Fig. 5).

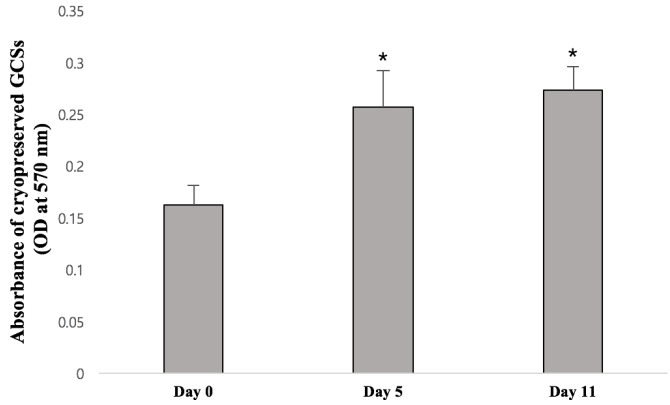

Figure 5. Alizarin Red S staining for mineralization of GCSs. Cryopreserved GCSs (gray) were measured on days 0, 5, and 11 of culture after thawing. Culture day 5 and 11 increased significantly compared to day 0 ($p < 0.05$).

IV. DISCUSSION

Slow-freezing of OCSs not only showed good cell viability but was also capable of producing a mineralized matrix at bone defect sites when comparing slow- and rapid-freezing methods (Kura *et al*., 2016). In the present study, I also used a slow-freezing method for cryopreservation of GCSs. In this study, I evaluated the effects of cryopreservation on the integrity of GCSs. Histological examination revealed that cryopreserved GCSs showed thinner cell layers compared with fresh GCSs, although the shape of the cell sheet remained intact after freeze-thawing. Cryopreservation of the osteogenic MSC matrix does not affect viability, osteogenic potential, or morphology (Xiang *et al*., 2007; Kotobuki *et al.,* 2005).

The viability of the cryopreserved GCSs immediately after thawing decreased to 76% that of fresh GCSs; however, this difference was not significant. Similarly, in a previous study, cryopreserved OCSs were found to show 70% viability compared with fresh OCSs (Kura *et al*., 2016). The cell proliferation rate decreased gradually until 2 days after thawing. In a previous study, the fresh GCSs showed significantly higher cell proliferation compared with OCSs, owing to activation of Wnt signaling (Kim *et al*., 2017). However, in the

present study, cryopreserved GCSs did not proliferate after freeze-thawing. Thus, these data suggest that freeze-thawing may have inactivated the Wnt pathway in GCSs.

β-catenin is an important factor of the canonical Wnt/β-catenin pathway (Zhang *et al*., 2010; Macsai *et al*., 2008). When this pathway is activated, it promotes the proliferation and differentiation of MSCs (Dravid *et al*., 2005; Gaur *et al*., 2005). β-catenin was not different between fresh and cryopreserved GCSs immediately after thawing. However, the expression of β-catenin decreased significantly at 2 days after thawing, which could indicate inactivation of the Wnt pathway.

The osteogenic differentiation process of MSCs consists of osteoprogenitor proliferation, matrix maturation, and mineralization (Owen *et al*., 1990). During the osteoprogenitor cell stage of MSC differentiation, Runx2 plays an important role in the early pathway of bone differentiation (Komori *et al*., 2005; Chen *et al*., 2012). Consistent with this, I found that Runx2 expression was significantly lower at 2 days after thawing and culture.

OPN is secreted in mature osteoblasts during the late stage of osteogenesis (Komori *et al*., 2005; Chen *et al*., 2012). Interestingly, in my study, I found that OPN expression was increased significantly at 2 days after thawing compared with that in fresh GCSs, suggesting that cells in GCSs differentiated to the late stage of osteogenesis after freeze-thawing. Additionally, BMPs play an important role in rapid bone formation and maturation as growth factors (Carpenter *et al*., 2010; Lavery *et al*., 2009). The mRNA expression of BMP-7 did not differ after freeze-thawing, indicating that GCSs maintained osteogenic ability regardless of whether the cell sheets underwent cryopreservation.

In the present study, initial osteogenic factors, such as Runx2 and β-catenin, increased in the GCSs immediately after thawing, but decreased after 2 days of culture. In contrast, late osteogenic factors, such as OPN, increased at 2 days of culture, and BMP-7 was maintained at a constant expression level after freeze-thawing. Thus, further cultivation of freeze-thawed GCSs could lead to later stages of osteodifferentiation. However, ARS showed that there were significant differences in mineralization from day 5, suggesting that the osteogenic differentiation process was stopped during freezing and proceed continuously after thawing.

Systemically injected cells migrate to the target site through the homing effect (Devine *et al*., 2003). Additionally, freeze-thawing has been reported to decrease the homing effects of MSCs (Chinnadurai *et al*., 2014). In contrast, cell sheets can be maintained in a localized region and function sufficiently without a sharp decrease in effect (Kura *et al*., 2016). Gelatin is a substance derived from collagen of the skin tissue and contains the arginine-glycine-aspartic acid (RGD) sequence. The RGD sequence promotes cell stability with the surrounding ECM (Hoch *et al*., 2012) and enhances cell adhesion through integrin (Wu *et al*., 2011; Rosellini *et al*., 2009). Thus, based on these characteristics of gelatin, GCSs are thought to be effective for cell adhesion, even if used right after thawing.

Collectively, my data demonstrated that the freeze-thawing process did not affect the osteogenic ability of GCSs and that GCSs could be used after freeze-thawing.

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VI. 국문 초록

개 지방유래 중간엽 줄기세포를 이용하여 만든 동결보존 젤라틴 골분화 세포시트의 골 형성능

지도 교수: 권 오 경

서울대학교 대학원

임상수의학 전공

정 태 성

젤라틴 골분화 세포시트(GCS)는 높은 골분화능을 보유하고 있으며 세포 시트 형성이 좋은 것으로 알려져 있다. 그러나 세포시트는 세포 준비 기간이 길기 때문에 임상 적용에 제한점이 있다. 이를 보완하기 위해 고안된 GCS 의 냉동보관은 세포 시트의 사용을 용이하게 할 수 있다. 본 연구에서는 동결-해동과정이 GCS 에

미치는 영향을 평가하였다. 신선한 GCS 는 풍부한 세포외기질을 형성하며, 시트의 두께가 3-4 층으로 두껍게 형성되는 것이 확인되었다. 그러나 동결-해동 후 시트의 두께는 1-2 층 정도로 감소하는 것인 확인되었다. 세포생존률의 경우 냉동 보관된 GCS 는 해동 직후 측정하였을 때 신선한 GCS 와 비교하여 유의적인 차이가 없음이 확인되었다. 그러나 동결 보존된 GCS 는 해동 후 재 배양 시 증식하지 않는 것이 관찰되었다. Runx-2 와 β-catenin 의 mRNA 발현 수준은 신선한 GCS 와 비교하였을 때 해동 직후에는 큰 차이가 없었지만, 2 일간 재 배양할 경우 유의적으로 낮아짐이 확인되었다(p <0.05). 그러나 osteopontin 의 mRNA 발현은 2 일간 재 배양할 경우 유의하게 증가하였다(p <0.05). BMP-7 의 발현은 냉동 유무 및 재 배양에 관계없이 높게 유지되는 것이 확인되었다. 또한 동결-해동 후 재 배양할 경우 칼슘 침착 정도가 지속적으로 증가되는 것이 확인되었다. 이러한 결과를 분석하였을 때 GCS 는 냉동 후에도 골 형성 및 시트 형태를 유지하는 능력이 존재함을 확인하였다. 이 기술을 통해 GCS 의 임상적 적용 시 제한점을 최소화 할 수 있을 것이다.

주요어: 젤라틴 골분화 세포시트, 냉동 보존, 골 형성 능력

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